Localization of Protein Disulfide Isomerase to the External Surface of the Platelet Plasma Membrane

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Protein disulfide isomerase (PDI) is an enzyme that catalyzes the formation as well as the isomerization of disulfide bonds. In this study, antibodies against PDI were used to show PDI antigen on the platelet surface by indirect immunofluorescence microscopy and by flow cytometry. The platelets were not activated, as evidenced by the absence of staining by an antibody against P-selectin. Permeabilized platelets showed little cytosolic PDI by indirect immunofluorescence microscopy, suggesting that the majority of platelet PDI is localized to the platelet surface. PDI activity against "scrambled" RNase was shown with intact platelets. The activity was inhibited by inhibitors of PDI and by an antibody against PDI. Other blood cells showed little PDI. Platelet surface PDI may play a role in the various physiological and pathophysiological processes in which platelets are involved.

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were conjugated to FITC as described elsewhere.7 The concentrations of FITC-labeled IgG were determined by the dye-binding assay using the unlabeled protein as a standard.8 The dye-to-antibody ratio was determined assuming a molar extinction coefficient at 495 nm for FITC of 65,000.9 The fluorescence/protein molar ratio ranged between 2 and 4 for the anti-PDI IgG and was 3 for the nonimmune IgG. FITC-labeled monoclonal mouse antiglycoprotein IX antibody (lgGk) as well as a phycocyanin-labeled monoclonal mouse anti-P-selectin antibody (clone AC12)12 were purchased from Becton Dickinson (San Jose, CA) and used at the manufacturer's recommended concentrations. Monoclonal FITC-lgGk specific for keyhole limpet hemocyanin (Becton Dickinson) and phycocyanin-lgGk (Amac, Westbrook, ME), which also recognizes a nonbiological hapten, were used as negative controls for the antiglycoprotein IX and anti-P-selectin antibodies.

Fab' of the rabbit anti-PDI antibody were prepared as described elsewhere,11 with minor modifications, by incubation of 600 µg of immune or nonimmune IgG with papain (10 µg) in 100 mmol/L sodium acetate, pH 5.5, containing 1 mmol/L EDTA, at 37°C for 4 hours. The reaction was stopped by the addition of iodoacetamide to a final concentration of 75 mmol/L for 30 minutes at room temperature. The Fab' were separated from the Fc portion by affinity chromatography over protein A. The concentration of the Fab' was determined by spectrophotometry at 280 nm using an extinction coefficient of 15.0.12

For the indirect immunofluorescence experiments, FITC-labeled goat antirabbit IgG was purchased from Cappel (Durham, NC). Human Fc fragments, FITC-conjugated affinity-purified goat antirabbit IgG, Fab', and fragment-specific IgG and FITC-conjugated affinity-purified F(ab')2 fragment goat antirabbit IgG, F(ab')2 fragment-specific (which reacts with the light chains on rabbit IgG and with Fab' fragment) were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Rabbit antihuman von Willebrand factor IgG was purchased from Celsus (Cincinnati, OH).

Cell preparation for the PDI assay and Western blotting. Blood was collected into 0.14 vol of 85 mmol/L citrate, 65 mmol/L citric acid, 2% dextrose (ACD) from normal aspirin-free adult volunteer donors through a 19-gauge needle using either no tourniquet or a loosely fitted tourniquet, and the first 2 mL of blood collected was discarded. Platelet-rich plasma (PRP) free of erythrocytes and leukocytes was prepared by centrifuging the whole blood at 300g for 20 minutes. PGE1 was added to a final concentration of 1 µmol/L to PRP, and the platelets were pelleted by centrifugation at 1,000g for 15 minutes at 22°C, then washed twice in phosphate-buffered saline (PBS; 10 mmol/L phosphate and 140 mmol/L NaCl, pH 7.4) containing 0.5 mmol/L EDTA, and resuspended in the same buffer. The buffer for the first wash contained 1 µmol/L PGE1; the second wash contained 300 mmol/L PGE1.

The erythrocytes for Western blotting were washed twice in PBS by centrifugation at 300g for 15 minutes. Polymorphonuclear leukocytes (PMNs) were prepared according to standard techniques.13 The number of platelets, erythrocytes, or PMNs in the suspension was
determined using a hemocytometer. The preparations of platelets and erythrocytes were determined to be greater than 99.9% pure, and the PMN preparation was 99% pure by examination of a Wrights-Geisma stain of the cells.

**PDI assay.** The assay was performed as described by Chen et al. In brief, PDI activity was detected by measuring the renaturation of RNase that had been inactivated by formation of randomly mismatched or scrambled disulfide bonds (scrambled RNase). In the presence of a low concentration of a thiol reagent (GSH), scrambled RNase renatures slowly. PDI accelerates this renaturation. Reactivated RNase was assayed by the digestion of RNA, which was measured by the increase in absorbance at 260 nm. Platelet PDI was assayed on a platelet-rich suspension (5 x 10^6 platelets/mL) maintained in a dispersed state by gentle pipetting during the 60-minute incubation period, after which the platelets were pelleted at 4,000 rpm in a microcentrifuge for 30 seconds at 22°C, and 10 μL of the supernatant fraction was added to the yeast RNA solution.

**Indirect immunofluorescence.** Platelets were obtained by collecting the blood into ACD solution and PRP was prepared as described. An aliquot of the PRP suspension was mixed with an equal volume of PBS containing 4% paraformaldehyde. After a 1-hour fixation at 4°C, the platelets were pelleted, washed in PBS containing 0.5 mM EDTA, and allowed to settle and dry on a glass slide. Peripheral blood smears from normal donors were prepared on glass slides. Smears of bone marrow aspirates were obtained from discarded preparations from patients who had undergone diagnostic bone marrow examination. These slides were fixed in 100% acetone at 4°C for 10 minutes. The slides were then incubated for 30 minutes with a blocking buffer containing 10% normal goat serum in PBS. This was followed by rabbit anti-PDI IgG or an equivalent concentration of nonimmune rabbit IgG for 45 minutes. The slides were then washed 3 times with PBS and incubated with the FITC-labeled goat antirabbit antibody for 45 minutes and washed again with PBS 3 times. As one approach to ensure that the IgGs were not binding to the platelet Fc receptors, for some experiments, the platelets were preincubated with human Fc fragments (10 μg/mL), and a secondary FITC-conjugated affinity-purified goat antirabbit IgG (Fab')2 was used.

**Immunofluorescence staining of unstimulated platelets.** The platelets were prepared by the technique of Wencel-Drake et al. Platelets prepared by washing as described in the cell preparation section (except 10 mM Tris and 150 mM NaCl, pH 7.4 [TBS], was used instead of PBS) or by gel filtration were fixed in 2% paraformaldehyde at 4°C for 1 hour. Unreacted aldehyde was blocked with 25 mM NH4Cl in TBS, and the platelets were permitted to settle on polylysine-coated glass slides. In some cases, the platelets were treated with 0.1% Triton X-100 for 3 minutes to render them permeable to antibody before staining. The permeable or intact platelets were then incubated for 45 minutes either with immune Fab' or antibody or with identical concentrations of nonimmune Fab' or antibody. The platelets were rinsed with TBS containing 0.1% bovine serum albumin and, when Fab' was used, stained for 45 minutes with FITC-labeled goat F(ab')2, antirabbit IgG Fab'. When the intact rabbit anti-PDI IgG was used, the platelets were stained with FITC-labeled goat antirabbit IgG. The slides were examined by light and fluorescent microscopy.

**RESULTS**

In this study, we used rabbit antiserum raised against purified platelet PDI for localization studies of PDI in platelets and megakaryocytes. The antiserum was passed through a human albumin-Sepharose affinity column and subsequently through a protein A column, from which IgG was eluted with 0.1 mol/L glycine at pH 3.0. The immune IgG was monospecific by Western blot, giving a single band corresponding to the molecular weight of purified human platelet PDI. In addition, a single line of identity was obtained by double immunodiffusion against solubilized platelet lysozyme and purified platelet PDI. The rabbit antihuman PDI IgG also bound to purified rat liver PDI and inhibited the activity of human PDI in the scrambled ribonuclease (RNase) assay.

**Localization of PDI in platelets.** Platelets were fixed in 2% paraformaldehyde in the ACD anticoagulated PRP and then incubated with the rabbit anti-PDI IgG. This was followed by reaction with a FITC-labeled anti-rabbit IgG antibody. Fluorescence was localized to the periphery or rim of the platelets (Fig 1A). Specificity was shown by using nonimmune rabbit IgG in place of the anti-PDI IgG; no staining was observed (Fig 1B). When platelets were fixed after washing (Fig 1C), a staining pattern similar to that obtained with platelets fixed in PRP was obtained, but no
staining was observed when a nonimmune IgG replaced anti-PDI IgG (data not shown). To further ensure that the binding of the immune IgG was not because of platelet Fc receptors, we performed studies on washed platelets using Fab' fragments made from both immune and nonimmune IgG. The results seen in Fig 1D show a peripheral fluorescence in the sample incubated with the anti-PDI Fab'. No fluorescence was observed with platelets incubated with identical concentrations of the nonimmune Fab' (Fig 1E). We conclude that the anti-PDI IgG binding to unstimulated platelets is specific for PDI on the platelet surface.

The flow cytometry analysis of PDI on platelets is shown in Fig 2. There was significant binding of the anti-PDI IgG to the platelet surface (Fig 2A) with minimal binding of the nonimmune rabbit IgG (Fig 2B). To determine whether PDI was on the surface only after platelet activation, we compared staining with an antibody to P-selectin, which is surface-expressed only after platelet activation. There was little fluorescence detected with the anti-P-selectin antibody, indicating that the platelets were not activated by the preparative procedure (Fig 2C). A positive control for this activation was PRP from the same donor's blood collected in 3.8% sodium citrate and activated with adenosine diphosphate (ADP; 10 µmol/L), which produced a substantial increase in P-selectin expression (not shown). Similar results were obtained using platelets from different donors. These studies indicate that resting platelets have PDI on the external surface.

To determine whether there may be an intracellular pool of PDI in platelets as well as that on the external surface, we studied platelets permeabilized with 0.1% Triton X-100. Figure 3A shows that the permeabilized platelets retain a peripheral fluorescent pattern but lack a cytoplasmic staining pattern. Platelets incubated with the nonimmune rabbit IgG showed no fluorescence (Fig 3B). When a rabbit anti-von Willebrand factor antibody was used a punctate staining pattern consistent with localization in alpha-granules was observed (Fig 3C). These studies indicate that the majority of the total platelet PDI pool is on the platelet surface.

**Assay for platelet PDI activity.** Because PDI is localized to the platelet surface, we examined PDI catalytic activity of platelets in suspension in the scrambled RNase assay. The PDI activity as a percentage of maximal renaturation of the RNase ranged from 18% to 27% in three experiments (Figs 4A through C). Three inhibitors of PDI reduced this activity to background level (Fig 4A), and anti-PDI IgG, but not nonimmune IgG, substantially inhibited the PDI activity (Fig 4B). Because it was possible that the activity measured in Fig 4 was not platelet-associated but was continuously released to the medium, platelets were maintained in suspension under the same assay conditions for 60 minutes at 22°C in the absence of GSH or scrambled RNase. After removal of the platelets by centrifugation, the supernatant solution as well as the resuspended pellet were assayed for PDI activity. Most of the PDI activity remained associated with the platelet pellet (Fig 4C). These data suggest an externally oriented PDI on intact platelets that has activity against a soluble substrate. The relatively small but significant amount of PDI activity in the supernatant solution raised the question whether this activity was associated with platelet vesicles or was soluble. To resolve this question the supernatant solution was subjected to centrifugation at higher centrifugal force (200,000g for 4 hours). Neither the activity nor the PDI protein (detected by Western blot) were pelleted suggesting that this PDI was released in a soluble form (see Discussion).

**PDI localization in megakaryocytes.** Indirect immunofluorescence of a bone marrow aspirate from two patients with acute lymphocytic leukemia in complete remission...
Fig 3. Platelet PDI by indirect immunofluorescence of permeabilized platelets. Unstimulated, fixed platelets were rendered permeable by treatment with Triton X-100. (A) The immunofluorescence pattern in platelets incubated with the anti-PDI IgG is shown. (B) Platelets incubated with nonimmune IgG are shown. (C) Platelets incubated with rabbit anti-von Willebrand factor IgG are shown. (Original magnification x1,000).

(Figs 5A and B) shows an intracellular pattern of fluorescence. Figure 5C is a higher magnification of a megakaryocyte. Figure 5D showed the negative control obtained with nonimmune rabbit IgG preparation.

Evaluation of other whole blood components for PDI. Indirect immunofluorescence of acetone-fixed peripheral blood smears were also analyzed using anti-PDI IgG or nonimmune IgG. Platelets showed substantial PDI with a location around the periphery, whereas erythrocytes, visible under light microscopy, were not fluorescent (data not shown). Western blot analysis using antiplatelet PDI IgG showed that platelets contain much more PDI per cell than erythrocytes (Fig 6A). No polymorphonuclear leukocyte PDI was detected (Fig 6B).

DISCUSSION

In this report, we present two lines of evidence that PDI is on the external surface of nonactivated platelets. The PDI activity of a suspension of intact platelets is much greater than the supernatant solution of the same suspension, and an antibody to PDI binds to the surface of intact platelets. Surface-binding was shown by both immunofluorescence microscopy and by flow cytometry. Absence of Fc-receptor binding in these experiments was shown by the failure of nonimmune IgG to stain and by the demonstration of staining with Fab' fragments. PDI was shown to be on the surface of resting platelets. We showed the absence of platelet activation by the absence on the external surface of a granule protein, P-selectin, known to be translocated to the surface on platelet activation.

There have been other reports of PDI on the external surface of mammalian cells. Akagi et al26 showed immunoreactive PDI on the plasma membrane of rat exocrine pancreatic cells, and Krishna Rao and Houseman27 isolated from chicken-embryo retina cell membranes a protein with 99% homology to chicken PDI. Mandel et al21 used inhibitors of
PDI activity (bacitracin and specific antibodies to PDI) to show PDI on the external surface of Chinese hamster ovary cells. An external location for PDI is, nevertheless, somewhat surprising, because current knowledge of PDI and its postulated functions all suggest an endoplasmic reticulum location. We have been unsuccessful in identifying PDI in the cytoplasm of platelets by immunofluorescence in conditions designed to permeabilize the platelet membranes. Although we cannot exclude small quantities of cytosolic PDI, the absence of a traditional endoplasmic reticulum in platelets and the absence of significant protein synthesis suggests that another location and function might be expected. The relative abundance of PDI in platelets compared with that in other blood cells studied would seem to further suggest a specific function of platelet PDI. It is interesting that PDI is diffusely located throughout the cytoplasm of megakaryocytes in relatively high concentration but is predominantly on the surface of platelets. PDI may serve as a valuable marker for studies of platelet formation from megakaryocytes, a process that remains to be clearly defined.
The previous demonstration that PDI activity was released from A23187 activated platelets14 left open the question of the source of the released PDI. This study suggests that it was derived from the surface, and the demonstration of PDI activity of intact platelets toward a soluble protein raises the question of whether surface-bound PDI on nonactivated platelets may not be as important as the released enzyme. In yeast and mammalian cells, PDI, which contains a C-terminal endoplasmic reticulum retention signal, is thought to bind to a specific receptor that facilitates its recycling from the Golgi apparatus back to the endoplasmic reticulum. In preliminary experiments using nonactivated platelets in suspension, PDI was released from the platelet surface with increasing pH in a manner consistent with the reported pH-dependent binding of PDI to its receptor.21,24 This potential for release of soluble PDI likely explains the findings of some PDI being released into the supernatant solution of nonactivated platelets (Fig 4C).

Potential functions of platelet PDI may be suggested. By labeling thrombospondin with thiol-reactive agents and subsequently performing thrombin digests on thrombospondin, we have been able to show that PDI can catalyze isomerization of internal disulfide bonds of thrombospondin (Huang et al, manuscript submitted). In this regard, the recent report of Sun et al25 showing that the cell adhesive abilities of thrombospondin depend on the state of its disulfide bonds suggests to us that PDI may effect the function of thrombospondin. Furthermore, Chen et al have provided evidence that platelet PDI is capable of disulfide cross-linking thrombin to thrombospondin. The processes of intramolecular disulfide exchange or intermolecular disulfide cross-linking of proteins could serve to modify the function of proteins or to localize them to the extracellular matrix. Because platelets are available at sites of vascular injury where a spectrum of hemostatic and tissue remodeling phenomena occur, other functions of platelet PDI could be proposed.

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